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Chip-based detection of hepatitis C virus using RNA aptamers that specifically bind to HCV core antigen

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Abstract

The development of reagents with high affinity and specificity to the antigens of hepatitis C virus (HCV) is important for the early stage diagnosis of its infection. Aptamers are short, single-stranded oligonucleotides with the ability to specifically recognize target molecules with high affinity. Herein, we report the selection of RNA aptamers that bind to the core antigen of HCV. High affinity aptamers were isolated from a 10^{15} random library of 60 mer RNAs using the SELEX procedure. Importantly, the selected aptamers specifically bound to the core antigen, but not to another HCV antigen, NS5, in a protein chip-based assay. Using these aptamers, we developed an aptamer-based biosensor for HCV diagnosis and detected the core antigen from HCV infected patients' sera with good specificity. This novel aptamer-based antigen detection sensor could be applied to the early diagnosis of HCV infection.

Keywords: Hepatitis C virus; SELEX; Aptamers; Biosensor; Diagnosis

The hepatitis C virus (HCV) is a single-stranded RNA virus which is responsible for chronic liver diseases, such as cirrhosis, end-stage liver disease, and hepatocellular carcinoma. Approximately 3% of the world's population—more than 170 million people—are infected with HCV [1]. The transfusion of HCV-contaminated blood is believed to be mainly responsible for the world-wide epidemic of HCV and, consequently, the accurate and sensitive diagnosis of HCV in blood samples is crucial.

At present, the most widely used method of diagnosing HCV is the detection of anti-HCV antibodies using a screening Enzyme-Linked Immunosorbent Assay (EIA) [2], based on recombinant proteins from the HCV genome. While it is highly sensitive and specific, this assay nevertheless has some limitations. For example, it cannot detect viruses during the early stage of infection, at which time antibodies against HCV antigens are not produced. In addition, patients who are immuno-suppressed following transplantation, or who are immuno-compromised secondary to infection with the human immunodeficiency virus (HIV), may exhibit HCV infection without having any detectable antibodies [3]. Moreover, the EIA sometimes generates false-positive or false-negative results [4]. While nucleic acid testing (NAT) methods are currently being used as supplementary tests, they are labor-intensive, expensive, and prone to contamination [5].

Another method of HCV diagnosis is to detect the HCV antigens present in serum using antibodies. Structural proteins such as the core antigen and the envelope glycoproteins E1 and E2 are suitable targets for the early stage

Abbreviations: HCV, hepatitis C virus; EIA, enzyme-linked immunosorbent assay; SELEX, systematic evolution of ligands by exponential enrichment; HIV, human immunodeficiency virus; NAT, nucleic acid testing; BSA, bovine serum antigen.

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diagnosis of HCV infection. However, due to the difficulty in generating high quality antibodies against HCV antigens, this method has not become feasible until very recently. Nonetheless, the recent development of a core antigen detection EIA kit (Trak-C, Ortho Clinical Diagnostics) [6] supports the concept of antigen diagnosis as an important addition to the current methods of antibody diagnosis. It also justifies the efforts that are being made to further develop antibodies or antibody-like reagents that can recognize HCV antigens with high sensitivity and specificity.

Aptamers are short, single-stranded oligonucleotides which can fold into specific three-dimensional structures in order to recognize target molecules such as small chemicals, proteins, or even cells [7,8]. High affinity aptamers for specific target molecules can be isolated from a library of randomized sequences in vitro by using the SELEX process [9–11]. Due to their thermal stability, long shelf-life, and ease of chemical synthesis and modification, in addition to their affinity and specificity which rival those of antibodies, aptamer-based biosensors are considered as promising molecular diagnostics of the future [12].

While several aptamers have been selected against HCV [13–17], none of these have been selected against structural proteins such as core or E1/E2 antigens. As the first step toward the development of aptamer-based HCV diagnosis, we selected RNA aptamers that can bind to the HCV core antigen with high affinity and specificity. The selected aptamers specifically recognized the core antigen, but not NS5, another HCV antigen. To our knowledge, this is the first report of aptamers recognizing the structural proteins of HCV. Furthermore, we applied the core antigen-specific aptamers to sol–gel based chips and detected the core antigens from HCV-infected patients' sera, thereby developing a novel HCV diagnostic method.

Materials and methods

Protein preparations. The HCV core and NS5 proteins [18,19] were cloned and expressed using the pET28 system (Novagen, Germany). BSA protein and anti-BSA antibodies were purchased from Sigma–Aldrich (USA). Cy3-labeled secondary antibodies (goat, rabbit, and mouse) were purchased from Abchem (UK).

SELEX. The synthesized SELEX library was purchased from Bioneer (Korea). The selection and amplification procedure was performed as previously described [20]. The progress of the selection process was monitored by means of the filter binding assay [20,21]. After nine rounds of selection, the aptamer pool was reverse-transcribed and then cloned into the vector, pGEM-3Z (Promega Inc., USA). The inserts in the individual clones were sequenced.

Aptamer synthesis. The selected aptamers (9-14, 9-15, 9-16, 9-17, and 9-19) were transcribed using a MEGAshortscript kit (Ambion, USA). In brief, after PCR amplifying the aptamer construct DNAs, 1 μg of the amplified templates was used for in vitro transcription according to the manufacturer's protocol. Fluorinated aptamers were made using the Durascribe transcription kit (Epicentre, USA) according to the manufacturer's recommendations.

Measurement of dissociation constants (K_ds). SPR analysis was performed using a BIAcore 3000 instrument. The proteins (HCV core proteins and thrombin proteins) were immobilized onto CM5 chips (BIAcore, Sweden). Five microliters of the HCV core proteins (70 μg/ml) and thrombin proteins (50 μg/ml) was injected with 100 mM sodium acetate (pH 4) for immobilization. The aptamers were diluted in HBS-N buffer [0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20] to 7 different concentrations: 30, 60, 120, 240, 480, 960, and 1920 nM. The chip surface was regenerated with an injection of RNase A (5 μg/ml, 20 μl, 2 μl/min). All assays were performed at 25 °C The association and dissociation rates and overall affinity (K_d = dissociation rate/association rate) were calculated using BIAevaluation 3.0 software (BIAcore, Sweden) using a 1:1 Langmuir binding model (A+B↔AB). Sensorgrams were transformed so that the injection points were aligned. Non-specific binding effects were subtracted using the sensorgram generated from flow cell 1, which was blocked without any proteins.

Protein–aptamer interactions on a 96-well plate based protein chip. Within the 8 mm diameter wells of 96-well plates (SPL, Korea), eight duplicate spots, including the 2 different C-type hepatitis marker proteins (HCV core, NS5) used in the standard EIA-based HCV assay, were printed along with negative controls (no protein) and positive controls (Cy-3 labeled proteins). Sol–gel protein chip printing methods were used as described previously [22]. Aptamers labeled with Cy-3 (Synthesized from Bioneer Co., Korea) were incubated for 30 min in each well and then washed using automated EIA equipment [23,24]. Then, the resulting plate chip wells were scanned and analyzed using a 96-well fluorescence scanner and the appropriate software program (FLA-5000 and Multi-gauge, Fuji Japan) [22]. The background intensity was subtracted from the signal intensity of each spot (LAU/mm^2).

Aptamer chip-based detection of HCV from human sera. Using the 96-well plate printing technology described above, 16 duplicated spots including 3 different HCV core protein aptamers (2′F-14, 15, and 19) which were fluorinated for RNase protection, C-type hepatitis marker proteins (HCV core and NS5), and HCV unrelated protein (BSA) were printed along with negative controls (no protein) and positive controls (Cy-3 labeled proteins). For the first set of 96-well plate chips (Fig. 4A), HCV core proteins and a core-specific antibody (purchased from Biodesigns, USA) were incubated for 30 min and, after washing, Cy3 labeled anti-goat antibody was added and the well plate chips further incubated. For the second set (Fig. 4B), sera from HCV positive and negative patients (obtained from the Korea University Hospital) and Cy-3 labeled human secondary antibody were incubated for 30 min in each well. Then, each set was washed using automated EIA equipment [23,24]. Finally, the wells were scanned and analyzed as described above.

Results and discussion

In vitro selection of RNA aptamers against HCV core antigen

HCV core antigen is a highly basic, viral nucleocapsid protein [18,19]. Several groups reported that the core antigen can interact with HCV and other infected disease antibodies and antigens non-specifically under certain conditions [25–28]. To obtain aptamers with high affinity and specificity to the HCV core antigen, we performed SELEX experiments against the HCV core antigen. A fusion protein including the N-terminal amino acids 2–114 of the core antigen with the C-terminal His tag was bacterially expressed, and the resulting core(2-114)-His6 proteins were purified using Ni-NTA affinity chromatography (Fig. 1A).

We then screened a 60 mer-randomized RNA library with 10^{15} complexity for core antigen binding using the SELEX procedure. After nine rounds of selection and

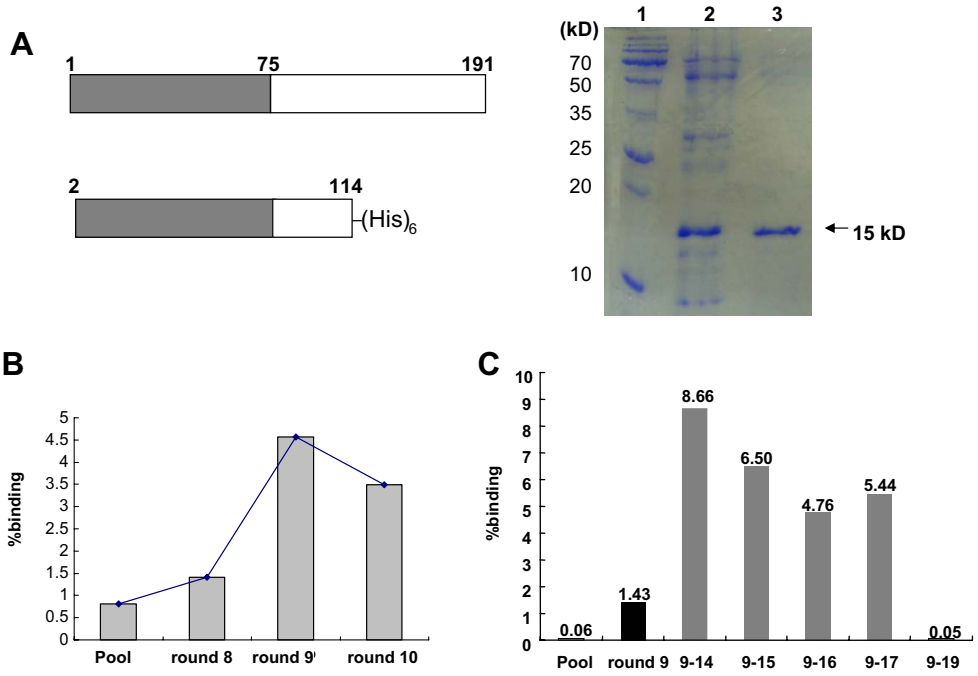

Fig. 1. SELEX targeting HCV core antigen. (A) Core protein structure, purification shown in Coomassie stained 15% SDS–PAGE gel. Lane 1: protein size markers, lane 2: crude extract, lane 3: purified core antigen. (B) Filter binding assays using 1% of selected transcripts after 8, 9 and 10 rounds of selection. (C) Filter binding assays of individual aptamers (% binding). 9-19 is a selected aptamer, but did not bind to core antigens in individual tests.

amplification, we observed the saturation of the binding (Fig. 1B). The individual RNAs were cloned and then tested for their core antigen binding. After testing dozens of individual RNAs, we identified the four RNA aptamers with the highest binding to the core antigen (Fig. 1C). No apparent similarity was observed between the high affinity aptamers in terms of their sequences and secondary structures as predicted by the Mfold program (Fig. 2 and Supplemental information 1). We further characterized the 9-14 and 9-15 aptamers, because they showed high affinity to the core antigen in both the filter binding assay (Fig. 1C) and the 96-well plate format protein chip assay

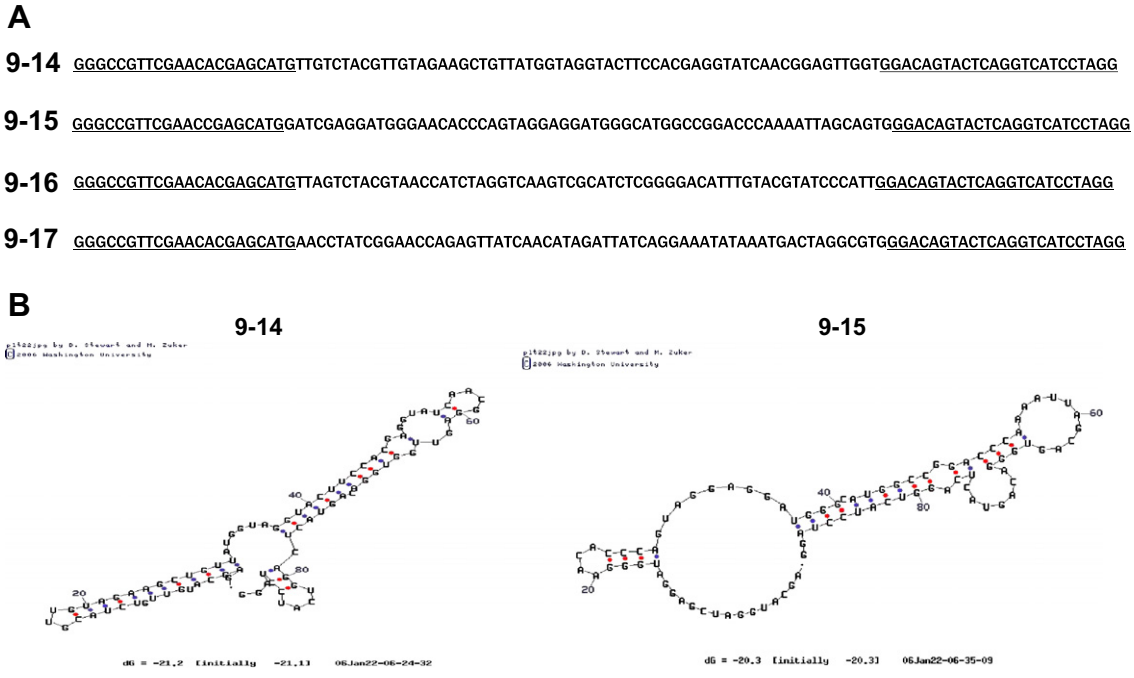

Fig. 2. (A) Sequences of selected aptamers and (B) secondary structure of aptamers 9-14 and 9-15 predicted by Mfold.

(Figs. 3 and 4, Supplemental information 2). The dissociation constants of the 9-14 and 9-15 aptamers were measured using the Biacore, and were found to be about 142 and 224 nM, respectively.

Specific detection of the HCV core antigen by RNA aptamers using a protein chip-based assay

To test the specific interactions of the aptamers with the HCV core antigen, we immobilized the core proteins using sol–gel based protein chips. This sol–gel based chip material has been used successfully for detecting antigen-antibody or protein–protein interactions with higher sensitivity than other currently available protein chip materials [22]. Especially, herein, we used 96-well plate format protein chips by printing 8 spots per well, including duplicates of the core and NS5 antigens along with negative and positive controls. After the automated incubation of each aptamer within each individual well, washing and scanning were done as described in the Materials and methods section. As shown in Fig. 3A, aptamers 9-14 and 9-15 showed specific interactions with the core antigen only, but not with the NS5 antigen, which is another HCV-expressed antigen. As expected, the non-binding aptamer 9-19 did not bind to any of the proteins.

We further checked the cross interactions of the aptamers with other proteins such as HCV NS3, E1/E2 antigens, HIV p24 antigens, and BSA proteins. However, we did not observe any interactions between the HCV core-binding aptamers and these proteins (data not shown). Therefore, it was confirmed that the selected aptamers 9-14 and 9-15 showed highly specific interactions with the HCV core antigen.

Aptamer biosensor for detecting hepatitis C virus antigens from virus-infected human sera

Using the core-specific aptamers, we developed an HCV biosensor prototype by immobilizing aptamers within the 96 well plates using the sol–gel-based immobilization method. Using the same sol–gel materials as those used for the protein chips [22], we immobilized the 2′-F modified (for protection of aptamers against RNases) aptamers (2′F-9-14 , 2′F-9-15, and 2′F-9-19) along with the core and NS5 proteins as diagnostic controls (Supplemental information 2). First, we checked for specific interactions between the aptamers immobilized on the chips and the incubated recombinant core antigens. After the incubation of the core antigen in each aptamer-printed well, the core antibody (goat) and Cy3-labeled goat secondary antibody were incubated. We were able to detect core-specific interactions with the 2′F-9-14 and 2′F-9-15 aptamers when compared with the negative control and the 2′F-9-19 control aptamer (Fig. 4A).

We then applied this 96 well format HCV biosensor prototype to human sera in order to determine whether we could diagnose HCV infection in real patients' samples. When we applied the HCV infected serum from an HCV patient along with the Cy3-labeled human antibody to the wells of the aptamer-immobilized HCV biosensor, the 2′F-9-14 and 2′F-9-15 aptamers showed stronger signals than the negative control and 2′F-9-19 aptamer (Fig. 4B). As well as the HCV patients' sera, we also applied a normal person's serum. The normal person's serum did not show any signal above the negative control from the core-binding aptamers (Supplemental Information 2). This result strongly suggests that this aptamer-based diagnosis method

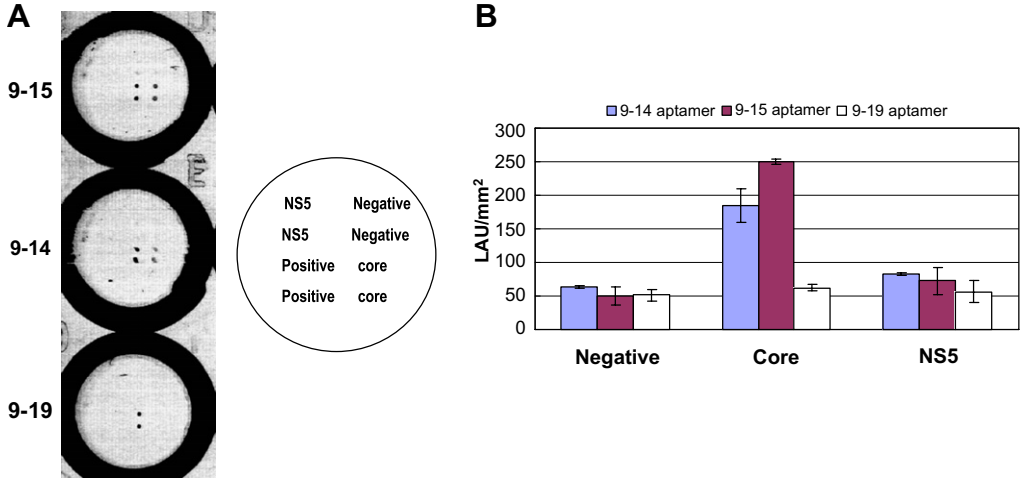

Fig. 3. Specificity of aptamers against HCV antigens. (A) HCV core and NS5 proteins with positive and negative controls were printed on each well of 96 well plate protein chips. The labeled aptamers (9-14, 9-15, and 9-19) were incubated in each individual well. After washing and scanning, specific interactions between immobilized proteins and incubated aptamers were shown as black dot signals. Aptamers 9-14 and 9-15 both interacted with only the core, not the NS5 antigens. However, 9-19 did not interact with any proteins. (B) The images in (A) were quantified using software as described in Materials and methods. After subtracting the background signal intensity from each spot signal, this background corrected signal intensity of each spot (negative, core, NS5) was plotted on the *Y*-axis (LAU/mm^2). Every chip experiment was done at least three times for the calculation of the standard deviations.

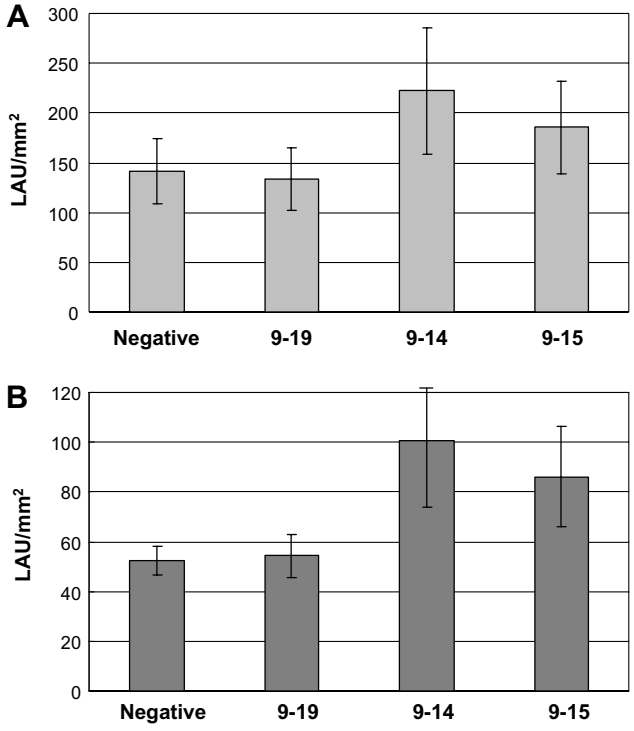

Fig. 4. HCV core antigen detection using aptamer-based biosensors. Aptamers 9-14, 9-15, and 9-19 along with negative controls were immobilized in each well of a 96 well chip (for complete images, see Supplemental information 2). In (A), each well was incubated with the core antigen and core antibody (goat) and washed, followed by incubation with the Cy3 labeled anti-goat antibody. In (B), each well was incubated with HCV infected serum from an HCV patient and Cy3 labeled-antibody (human). The resulting wells were scanned and analyzed in order to quantify the specific interaction signals (measured by intensity LAU/mm^2) between the immobilized aptamers (*X*-axis) and incubated core antigens from the purified proteins (A) or human sera (B). We analyzed at least 10 wells or chip experiments to confirm the reproducibility of the results.

can specifically detect the core antigens from HCV infected patients' sera.

Compared with the protein chip signals in Fig. 3 (~4–5-fold higher signals than the negative and 9-19 control signals), the aptamer chips showed weaker signals (~2-fold higher signals than the negative and 9-19 control signals) in Fig. 4. One reason for the reduced signals in the aptamer-immobilized chips could be the fact that the sol–gel formulation used for immobilization was optimized for proteins, not for nucleic acids. However, we still observed consistently higher signals from the 2′F-9-14 and 2′F-9-15 aptamer spots (Fig. 4B), which reflect the specific interactions between the aptamers and core antigens in either purified form or in HCV-infected serum.

In summary, we selected aptamers that can specifically recognize the HCV core antigen, but not the NS5 antigen. We demonstrated that these aptamers can specifically interact with the core antigen imbedded within the sol–gel chips. Then, this sol–gel formulation was used to immobilize aptamers within 96 well plates in order to generate an aptamer-based biosensor prototype for HCV diagnosis.

Herein, the affinity of the selected aptamers measured by the Biacore was around 100 nM, which is consistent with the K_d values measured with filter binding methods (data not shown). In addition, the K_d values of these aptamers were only 10-fold lower than those of the non-binding aptamer to the core antigen (~1.64 μM). The relatively high affinity of the core antigen to the non-binding aptamer could be due to the fact that the HCV core antigen is a basic nucleic acid binding protein which has some non-specific binding affinity to nucleic acids in general. Despite the relatively small differences in the K_d values between the core-binding aptamers and non-binding aptamers, the core-binding aptamers showed high avidity (data not shown), which could explain the consistently stronger signals observed in the chip-based assay in Figs. 3 and 4.

In order to generate a more sensitive aptamer chip for HCV diagnosis, the selected aptamers need to be further optimized. For example, the minimal domain required for the interaction with the HCV core antigen could be identified and then multimerized to further increase the avidity. Base modifications such as 2′-F or 2′-OMe should be introduced in order to confer resistance to nucleases present in the patient's serum. Once optimized, we believe that the aptamer chip generated in this manner could be used for the sensitive and specific detection of multiple HCV antigens in patients' blood samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.057.

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